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Small GTPases Take the Stage

Meeting Review

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Small GTPases are molecular switches that have been adopted to control many eukaryotic cell functions. Starting with the study of the protooncogene Ras in the early 1980s, detailed pathways have been uncovered upstream and downstream of Ras-related GTP binding proteins. Nonetheless, novel members have been discovered at a pace that has outstripped cell biologists, and thus much remains to be established regarding newer family members. Undiscovered functions are still being uncovered for "established" small GTPases such as Ras, Rho, and Ran. The topics covered at this meeting indeed demonstrate that Ras proteins are at the heart of cellular dynamics.

The recent FASEB meeting "Small G Proteins and Cell Dynamics" took place on July 6–11, 2002 in Snowmass, Colorado. In his opening introduction, Ian Macara pointed to the tremendous expansion of the small GTP binding protein superfamily since the discovery of the viral Ras oncogenes in the early 1980s. Ras initially came under close scrutiny once it was realized that mutations in Ras are common in human cancers. The first hints that these small (~200 residue) GTP binding proteins might be present in different forms was the discovery of a Ras homologous (Rho) gene in *Aplysia* by Pascal Madaule in 1985 and the realization that such proteins are ubiquitous (Madaule and Axel, 1985). It was apparent even at this stage that common themes among these proteins included membrane targeting sequences and conservation of amino acids involved in GTP binding. Following the completion of various genome projects, it is clear that all distinct subfamilies of small G proteins are found in eukaryotes, though specific proteins are not always represented. This meeting covered the Ras, Rho, Rab, Sar1, Ran, and Arf subfamilies. The organizers, Linda Van Aelst and Pierre Chardin, broke with the convention of segregating the sessions based on each GTPase subfamily to provide for a more holistic approach. Emerging themes were the extensive crosstalk between small GTPases, allowing coordination of different aspects of cellular function: new information on the way the microtubule network is coordinated, and the analysis of protein and signaling dynamics in live cells. Here, I summarize these areas; apologies must go to the excellent speakers who are not mentioned due to space constraints.

Seeing Is Believing

Small GTP binding proteins have no intrinsic catalytic activity, but exhibit selective binding to other proteins when in their active GTP-bound form. The nucleotide binding state, and thus activity, of most small GTPases is regulated by a variety of interacting factors such as GTPase activating proteins (GAPs) and GTP/GDP exchange factors (GEFs). To observe the cellular activation status of these proteins, researchers have developed a number of elegant fluorescent constructs that report the bound GTP status of various small GTPases. Mark Philips (New York University School of Medicine) showed how studying the behavior of Ras using green fluorescent protein (GFP) coupled to the Ras binding domain of Raf can lead to new insights about Ras signaling. Philips described how, in addition to the expected plasma membrane signal, oncogenic H-Ras and N-Ras engage Raf-1 on the Golgi and that endogenous Ras and unpalmitoylated H-Ras are activated in response to mitogens on the Golgi and endoplasmic reticulum (Chiu et al., 2002). H-Ras.GTP that is restricted to the ER can nonetheless activate the Erk pathway and transform fibroblasts, while Ras localized on other membrane compartments differentially engages downstream signaling pathways. Thus, Ras signaling is not limited to the plasma membrane as current models suggest, but also proceeds on endomembranes.

Michyuki Matsuda (Osaka University) described the range of probes that they have developed to study in real-time Ras, Rap, and Rho signaling, and their potential pitfalls. Their previous studies in PC12 cells used FRAP (fluorescence recovery after photobleaching) to reveal that Ras.GTP activity at neurites was due to high GTP/GDP exchange rate and/or low GTPase activity, but not to the presence of stable Ras.GTP (Mochizuki et al., 2001). Newer probes, designated Raichu-Rac and Raichu-Cdc42, consist of a Cdc42 and Rac binding domain of PAK, Rac1, or Cdc42, a pair of green fluorescent protein mutants, and a membrane binding CAAX box of Ki-Ras. With these, Rac.GTP and Cdc42.GTP production were monitored in motile HT1080 cells, where both were found to appear toward the leading edge and decreased rapidly when cells changed direction (Itoh et al., 2002). At higher magnification, Rac activity concentrated immediately behind the leading edge, whereas Cdc42 activity was most prominent at the tip of the leading edge, coincident with the generation of corresponding actin structures (lamellipodia and filopodia).

Klaus Hahn was one of the first researchers to investigate the activation of Rac in vivo (Kraynov et al., 2000). More recently, he has pioneered potentially less invasive approaches by attaching "push-pull" dyes onto relevant signaling proteins (Hahn and Toutchkine, 2002). The environmental change upon activation can lead to significant changes in fluorophore emission (in some cases, 3-fold increases), which can be quantified relative to a different control soluble reporter (GFP). Using a number of examples, he described how posttranslational modifications, ligand interactions, and conformational changes can be monitored in single cells. Such imaging techniques that probe the spatial resolution of signaling pathways promise to bring new understanding to old signal transduction pathways.

Observation of the formation of vesicle coats in real

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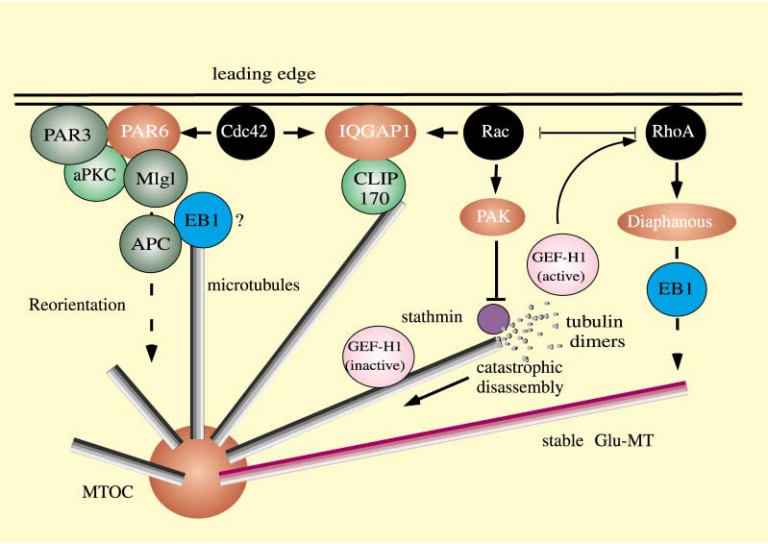

Regulation of Microtubule Dynamics by Rho GTPases

The Rho effector diaphanous (mDia) not only organizes actin but also causes stabilization of microtubules, which are marked by detyrosination, exposing the C-terminal glutamate (Glu-MT). Cellular MTs undergo a natural catastrophic disassembly, a process that can be promoted by protein factors such as stathmin; as indicated, its activity is negatively regulated by PAK phosphorylation downstream of Rac. MT disassembly leads to Rho activation via GEF-H1, which becomes active upon release. Both IQGAP and mPAR6 proteins directly bind to Cdc42.GTP, the latter through its complex with atypical PKCξ, which leads to reorientation of the MTOC, whose position aligns to the direction of cell movement. This might involve EB1, which localizes to MT plus ends. The mPAR6 also binds Mlgl. The IQGAP/CLIP170 complex is proposed to provide the local environment for capturing and stabilization of MTs at the leading edge.

time was described by Bruno Antonny (PIMC-CNRS). These processes involve the use of purified yeast protein components Sec23/24p and Sec 13/31p to monitor the assembly of COPII coats onto synthetic liposomes, as monitored by light scattering (Antonny et al., 2001). Only when Sar1p.GTP was bound to the liposome could a single round of assembly and disassembly of the COPII coat take place. The two COPII complexes, Sec23/24p and Sec13/31p, bind almost instantaneously (in less than 1 s) to such Sar1p.GTP-primed liposomes. Binding was followed by a fast disassembly (over a period of less than 10 s), due to a 10-fold acceleration of the GTPase activating protein activity of Sec23/24p by the Sec13/31p complex. Sec23/24p may in fact provide residues to promote GTP hydrolysis on Sar1p. This combination of biophysical techniques and the use of purified proteins demonstrates how membrane trafficking events can be reconstituted and followed in vitro.

Microtubules Fall Under Small G Protein Control

Microtubules (MTs) are composed of tubulin heterodimer subunits that assemble predominantly at one end (the plus end) from GTP-primed tubulin in the cytoplasm. As for actin filaments, assembly is controlled by a variety of proteins that decorate the surface of the MTs. During mitosis, the formation of microtubule spindles is critical for correct segregation of chromosomes. The small GTPase Ran is a key regulator of nucleocytoplasmic transport during interphase. Asymmetric distribution of the GTP-bound form of Ran across the nuclear envelope determines the directionality of many nuclear transport processes. Recent findings that Ran also functions in spindle formation and nuclear envelope assembly during mitosis (Ohba et al., 1999) suggest that Ran has a general role in chromatin-centered processes. Ian Mattaj reviewed how Ran affects these processes at many levels (see Hetzer et al., 2002). The action of Ran.GTP in spindle formation requires TPX2, a microtubule-associated protein previously known to target a motor protein, Xklp2, to MTs. TPX2 is normally inactivated by binding to the nuclear import factor importin α, and is displaced by the action of Ran.GTP. This release of TPX2 from a sequestering complex mirrors Ran function during transport (Gruss et al., 2001). He also discussed how manipulation of TPX2 by siRNA could similarly affect spindle assembly in living cells.

For some 10 years, researchers have focused on the role of Rho GTPases in the control of actin (and to a lesser extent intermediate) filament networks. Interestingly, a number of new studies demonstrate that Rho GTPases can regulate the microtubule cytoskeleton (see Figure). In cultured mammalian cells, MT minus ends are anchored at the microtubule organizing center (MTOC), and alternate between phases of growth and catastrophic disassembly (Desai and Mitchison, 1997). Targeting and capture of microtubule plus ends at special cortical regions are essential for polarized processes such as cell migration and axonal outgrowth. Work from the laboratory of Kozo Kaibuchi (Nagoya University) was described by Masaki Fukata. They have recently found that an effector known to bind Cdc42 and Rac, IQGAP1, is associated with the protein CLIP170, which in turn binds to the growing end of microtubules (Fukata et al., 2002). Genetic analysis in fission yeast has already shown that in the absence of tip1p, the CLIP-170 homolog, MT plus ends become destabilized uniformly throughout the cell, whereas Tip1p-positive (wild-type) cells exhibit this effect only when MTs reach the cell tip (Brunner and Nurse, 2000). Significantly, the mammalian IQGAP1 protein can be cosedimented with microtubules, and microinjection of a C-terminal fragment of IQGAP1 can disrupt the normal localization of CLIP-170 in Vero cells. Because Cdc42 and Rac1 are activated at the front of migrating cells, this complex of IQGAP/CLIP-170 is proposed to provide a signal that allows capturing and stabilization of the plus end of MTs (see Figure). In addition to CLIP-170, Tirnauer and Bierer (Tirnauer and Bierer, 2000) have reported that EB1 similarly localizes to the plus ends of growing MTs. This EB1 was originally identified as a protein that interacts with adenamatous polyposis coli (APC) tumor suppressor (Su et al., 1995). APC in turn binds to and activates a Rac1 GEF known as

Asef (Kawasaki et al., 2000), suggesting a local signaling loop that feeds back into Rac1.

Two other speakers touched on the behavior of the MT network in relation to EB1 function. Sandrine Etienne-Manneville discussed work carried out in Alan Hall's laboratory (University College London). Her studies of primary astrocyte migration in a wounded monolayer of cells suggested that Cdc42 responds to an integrin-mediated signal at the "open" side of the wound to affect two processes (Etienne-Manneville and Hall, 2001). First, a resultant Rac/PAK signal leads to local membrane protrusion associated with cell elongation. Second, the interaction of Cdc42 with the complex Par6/Par3/aPKC drives polarization of the MTOC to face the direction of cell elongation. This process can be blocked by inhibitors of atypical aPKC. They have also found that this pathway includes the components dynein and dynactin, which are principal proteins in MT capture and sliding in yeast, though direct interaction of the Par6 complex with these is not established.

Biochemical details of this complex of Cdc42/Par6/Par3/aPKC proteins were given by Ian Macara (University of Virginia) who was one of the first researchers to identify Par6 as a Cdc42 effector (Joberty et al., 2000). He also presented a recently obtained crystal structure of the Cdc42/Par6 complex. In epithelial cells, this complex localizes to tight junctions where it is implicated in the correct formation of these junctions at epithelial cell-cell contacts. New results presented by Pamela Plant (SLRI, Mount Sinai Hospital) indicate that the homolog of the tumor suppressor lethal giant larvae (Mlgl) is also targeted to Par6C and becomes phosphorylated by aPKC. Consistent with this are findings that related tumor suppressor genes *lgl* and *dlg* regulate basal protein targeting in *Drosophila* neuroblasts (Peng et al., 2000). It looks as if the mPar6 complex is going to get more complicated.

According to Gregg Gundersen (Columbia University), regulation of MT networks occurs by two separate mechanisms involving Rho GTPases. First, the positioning of the MTOC toward the direction of cell migration requires Cdc42 activity and the dynein/dynactin complex (Palazzo et al., 2001b), while the formation of stable MTs within the cell depends on RhoA and its effector mDia (Palazzo et al., 2001a). These stable MTs contain tubulin that has been C-terminally detyrosinated, and this allows them to be marked by a specific antibody. Using information already gleaned regarding the genetics of MT capture in budding yeast, they have hypothesized in a recent review that EB1 might form a link between the formin mDia and microtubules (Gunderson, 2002). Consistent with this, they have been able to show that the C terminus of EB1, which binds to APC, could block RhoA-mediated formation of stable MTs. Additionally, APC itself was capable of generating antibody reactive stable MTs.

Gary Bokoch (Scripps Research Institute) presented some beautiful time-lapse images of the way in which inhibiting the kinase PAK can change the real-time dynamics of MTs. A number of previous publications had suggested such a link: for example, the MT-destabilizing capacity of stathmin is blocked by PAK phosphorylation of serine16 (Daub et al., 2001), suggesting that PAK functions to locally stabilize MTs. A newer member, PAK5, has been shown to have similar MT-stabilizing properties (Cau et al., 2001).

Interestingly, not only do Rho GTPases regulate the MT network, but the converse is also true (see Figure). Dr. Bokoch described how the RhoA exchange factor GEF-H1 mediates crosstalk between microtubules and the actin cytoskeleton (Krendel et al., 2002). GEF-H1 mutants that are deficient in microtubule binding have higher activity levels than wild-type microtubule-bound forms and cause Rho-induced changes in cell morphology and actin organization. Conversely, changes in the actin cytoskeleton induced by MT depolymerization are blocked in part by nonactive versions of GEF-H1.

Interconnections among Small G Proteins

While crosstalk between small G proteins of the Ras and Rho family have long been described (Ridley et al., 1992), it is only recently that mediators have come to light. That connections exist between other small G proteins is to be expected and is coming under increasing scrutiny.

Ivan de Curtis (DIBIT, San Raffaele Scientific Institute) described his studies of a Rac1-associated complex that contains PAK, PIX, and an ArfGAP known as APP1/GIT1 (Di Cesare et al., 2000). He found that this APP1—which is believed to act on the small GTPase Arf6—accumulates on Rab11-containing recycling vesicles (Matafora et al., 2001). Interestingly, Rac1N17 (a dominant interfering mutant of Rac1), but not the active RacV12, colocalizes with these APP1-containing endosomes. Thus, formation of membrane ruffles by Rac1 is likely to be accompanied by the facilitation of membrane recycling via Arfs; this makes perfect sense in terms of stimulating membrane dynamics and actin-containing structures underlying lamellipodia.

Jim Casanova (University of Virginia) has also been looking at the function of Arf6 in some detail via its upstream regulator ARNO (Arf nucleotide binding site opener). ARNO acting via Arf6 induces Madin-Darby canine kidney epithelial cells to develop broad lamellipodia, separate from neighboring cells, and migrate (Santy and Casanova, 2001). Interestingly, ARNO not only activates Arf6, but also increases the level of Rac.GTP. This activation of ARF6 also results in increased activation of phospholipase D (PLD), and inhibition of PLD inhibits motility, though strangely it does not prevent activation of Rac. How might this Rac activation occur? One possible link lies between Arf6 and GIT1/PIX (a Rac1 activator), and indeed ARNO synergizes with PIX in the formation of large lamellipodia, an effect not seen with a mutant of PIX that is inactive toward Rac1. Both PIX and GIT colocalize with ARNO in cells, and that ARNO-induced motility was substantially inhibited by either a mutant GIT that cannot bind PIX or a PIX mutant that has no catalytic activity, suggests that PIX may mediate the activation of Rac downstream of Arf activation.

Hollis Cline (Cold Spring Harbor Laboratory) has studied GTPase function in the context of neuronal development in live *Xenopus* brain. Using a combination of single-cell microinjection, confocal microscopy of living brain, and a novel Rho GTPase assay, she recently demonstrated that crosstalk between Rho GTPases affects

dendritic arbor formation in vivo (Li et al., 2002b). RhoA activity is increased by activated Rac1 or by inhibition of Cdc42, while Rac1.GTP formation is inhibited by activated RhoA. It is suspected, but not proven, that this occurs through modulation of Rho GEFs. More recent work shows how these processes in the optic tectum are initiated by light stimulation and mediated via NMDA receptors. This produces an increase in dendritic branching and extension of these branches, thus increasing the dendritic field. Using a combination of protocols, she showed how optic nerve stimulation increases in Rac.GTP and decreases RhoA signaling.

The first activator of Ras to be described in mammals belonged to the son-of-sevenless (Sos) family. This protein is somewhat typical of Ras (and Rho) guanine nucleotide exchange factors (GEFs) in that it has a large multidomain structure, including a proline-rich C-terminal region that binds to the adaptor Grb2. Pier Paolo Di Fiore (European Institute of Oncology) has been studying other interactions of Sos. Signaling from receptor tyrosine kinases has long been known to result in sequential activation of the small GTPases Ras and Rac. Sos can be engaged in a tricomplex with Eps8 and E3b1/Abi-1, an RTK substrate and an adaptor protein, respectively. His group has shown that Sos-1, E3b1, and Eps8 assemble into a trimeric complex, while Grb2 and E3b1 bind through their respective SH3 domains to the same site on Sos-1 (Innocenti et al., 2002). This suggests a switch to determine whether Sos is endowed with Ras- and Rac-specific GEF activities. Interestingly, while the Sos-1/Grb2 complex is disrupted upon RTK activation, the Sos/E3bi/Eps8 complex is not. This is consistent with an activation of Ras that is short lived, while activation of Rac is more sustained. Di Fiore suggests that the complex can also recruit the p85 subunit of phosphatidylinositol 3-kinase (PI[3]K), which has been implicated previously in Rac1 activation (Rodriguez-Viciana et al., 1997). Nimnual and Bar-Sagi recently discussed how these two activities of Sos can be regulated via adaptor proteins Grb2 and E3b1 (Nimnual and Bar-Sagi, 2002).

Other modes of crosstalk between Ras and related small GTPases were covered by Channing Der (University of North Carolina). Through a bioinformatics screen and experimental verification, his group has shown that Tiam1, a Rac-specific GEF, binds activated Ras through a Ras binding domain (Lambert et al., 2002). Furthermore, activated Ras and Tiam1 cooperate to cause synergistic formation of Rac.GTP in a PI(3)K-independent manner. Thus, in cell systems that express Tiam1, this Ras effector can directly mediate Ras activation of Rac. Channing Der also presented surprising findings on the pathways downstream from Ras that lead to cell transformation (Hamad et al., 2002). Ras stimulates primarily three main classes of effector proteins, Raf kinases, PI(3)K, and RalGEFs (activator of Ral small GTP binding protein), with Raf generally being the most potent at transforming murine cells. Using oncogenic Ras mutants that activate single effectors as well as constitutively active effectors, Der and colleagues found that RalGEF, and not the Raf or PI(3)K pathway, is sufficient for Ras transformation in human cells. Thus, oncogenic Ras may transform murine and human cells by distinct mechanisms, with the RalGEF pathway a new target for anticancer therapy.

John Collard (Netherlands Cancer Institute) described how the analysis of Tiam1 knockout mice can be used to probe the role of Rac1 in cell transformation. Tiam1 is a Rac1 GEF that was cloned on the basis of its ability to promote lymphoma cell invasion (Habets et al., 1994). *Tiam1*$^{-/-}$ mice develop normally, even into old age; however the strong expression of Tiam1 in certain basal epithelial cells led the team to investigate whether there were differences in the potential to progress to cell transformation. *Tiam1*$^{-/-}$ mice were indeed found to be much less susceptible to tumor initiation and promotion based on the skin model using the application of the mutagen DMBA followed by multiple applications of a tumor promoter TPA (Malliri et al., 2002). The decrease in tumor induction could be explained by an increase in apoptosis, which probably removes many of the cells in the initiation phase. This selective pressure in turn may cause the few tumors that form in the *Tiam*$^{-/-}$ mice to be more aggressive. Such studies suggest that a mechanism exists by which Rac1 is protective against apoptosis, although the mechanism here is yet to be uncovered.

Where and When Are GTPase Effectors Activated?

Given the abundance of GTPase effector proteins that are being uncovered, the issue of target selection is bound to be of increasing significance. Martin Schwartz (Scripps Research Institute) described his efforts to discover why Rac1.GTP only associates with one of its effectors, PAK1, under certain conditions. Previously, his group showed that active Rac.GTP does not interact with PAK1 if cells are detached from the extracellular matrix (del Pozo et al., 2000). Movement of Rac.GTP to membranes is independent of effector interactions, but instead requires the polybasic sequence near the Rac carboxyl terminus that are involved with binding RhoGDI (del Pozo et al., 2002). A FRET-based study surprisingly demonstrated that, despite its uniform distribution, the interaction of activated RacV12 with a soluble PAK domain occurs predominantly at specific regions near cell edges, and is induced locally by integrin stimulation.

Finally, the use of phospho-specific antibodies was demonstrated to answer questions regarding the temporal and spatial activation of kinases downstream of small GTPases. Using an antibody against a CDK5 site previously identified in PAK1, Jonathan Chernoff (Fox Chase Cancer Centre) described how PAK1 is regulated through the cell cycle by Cdc2 (Thiel et al., 2002). Cdc2 phosphorylates PAK1 at serine 212, and inhibition of Cdc2 abolishes Pak1 mitotic phosphorylation in vivo. Expression of a PAK1 mutant in which Thr 212 is replaced with a phosphomimic (aspartic acid) has marked effects on the rate and extent of postmitotic spreading of fibroblasts. Although this phosphorylation does not affect the activity of PAK1, recent findings using different antibodies prepared against the phosphorylated activation loop of PAK1 similarly indicate that the kinase is active in mitosis (Li et al., 2002a). A mechanism involving Cdc42 or Rac activation of PAK and subsequent recruitment to the nucleus is suggested but remains to be proven.

Conclusion

The hunt for function among small GTPases is by no means complete, and it is only through the combination of model systems, high-resolution imaging, biochemistry, and dogged cell biology that we can take our understanding to the next level. Certainly, the behavior of these molecular switches will often be cell context dependent and, as Bill Balch emphasized, function must be sought in the correct context or the experimenter will be searching in vain. The advent of "big science" in the form of genome projects and microarray analysis is certainly an aid to this end. I suspect that this FASEB meeting will be running for some time.

Acknowledgments

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